

Clindamycin-Induced Enterocolitis in Hamsters as a Model of Pseudomembranous Colitis in Patients

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Stools from a patient with antibiotic-associated colitis and cecal contents from a hamster with clindamycin-induced enterocolitis were compared in a cytotoxicity assay to determine common properties. Both specimens produced actinomorphous changes in human amnion cells at 10^{-7} dilutions. The toxin was acid labile, heat labile, nonether extractable, non-dialyzable, and produced maximum activity at 60% with ammonium sulfate precipitation. Cytotoxicity was neutralized with clostridial antitoxin but not with equine serum. *Clostridium difficile* was recovered in high concentrations in specimens from both the hamster and patient. The supernatants of these *C. difficile* strains produced cytotoxic effects which were also neutralized by clostridial antitoxins. These results indicate that clindamycin-induced enterocolitis in hamsters is a model of human disease and implicate toxin-producing clostridia as responsible agents.

Pseudomembranous colitis (PMC) is a serious intestinal disease which has been found in a variety of clinical settings (1). Despite extensive research, no etiological mechanism has been clearly established and no specific therapeutic modality has been found to be consistently effective. A suitable animal model would facilitate such studies. Several investigators are currently studying clindamycin-associated colitis in hamsters with the hope that the lessons learned will be applicable to the clinical setting (1, 6; J. G. Bartlett, A. B. Onderdonk, and R. L. Cisneros, *Gastroenterology*, in press). However, there has been reluctance to accept the hamster as a model of human disease, since it is known that many small laboratory animals develop a similar form of lethal enterocolitis when exposed to a variety of antimicrobials (3).

A recent report by Larson et al. showed that stools from patients with antibiotic-associated PMC were cytopathic in tissue culture (5). The authors postulated that a toxin was responsible, but the source and nature of this toxin was not pursued. Recent work from our laboratory showed that clindamycin administration to hamsters resulted in high concentrations of *Clostridium difficile* in cecal contents of animals which expired with enterocolitis. Injections of whole cells or the cell-free broth supernatant of this organism produced lethal enterocolitis when introduced intracably to other animals (J. G.

Bartlett, A. B. Onderdonk, R. L. Cisneros, and D. L. Kasper, *J. Infect. Dis.*, in press). This work suggested that a clostridial toxin was responsible for clindamycin-induced enterocolitis in hamsters.

The present report employs a cytotoxicity assay similar to that described by Larson et al. to test specimens of stools from a patient with PMC and cecal contents from a hamster with clindamycin-induced enterocolitis. The purpose was to compare the disease in humans and hamsters to detect common properties.

MATERIALS AND METHODS

Source of hamster cecal contents. A 100-gm male Syrian hamster (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) was given three daily 2-mg intramuscular injections of clindamycin phosphate (The Upjohn Co., Kalamazoo, Mich.). The animal expired on day 4 after the initial dose, and necropsy showed a dilated hemorrhagic cecum with microscopic changes as previously described (Bartlett et al., *Gastroenterology*, in press). Cecal fluid was aspirated and retained at -40°C for subsequent experiments.

Source of human stools. A 56-year-old man underwent a sigmoid colectomy for colonic carcinoma. He received 12 doses of parenteral cephalothin (Eli Lilly & Co., Indianapolis, Ind.) as prophylaxis in the perioperative period. On day 6 postoperation he developed diarrhea, a distended abdomen, and hypotension. Re-exploration showed an intact anastomosis and no evidence of intra-abdominal sepsis. After the second operation, diarrhea fluid was collected and retained at -40°C for subsequent testing. Two days

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later the patient expired, and an autopsy showed PMC involving the cecum, colon, and rectum.

Cytotoxicity assay. The stool and cecal specimens were treated with an antibiotic mixture which is routinely used for processing stool samples for viral cultures and then centrifuged at $2,000 \times g$ for 20 min. Serial 10-fold dilutions were made of the supernatants with phosphate-buffered saline, and 0.1-ml portions of each dilution were inoculated into the cell cultures. The cultures were examined microscopically at hourly intervals for 3 and then daily for 3 days. Initial studies were performed with human amnion, WI-38, and BHK cell cultures. Primary human amnion cell cultures were prepared by trypsinization of fresh human amnion and grown in 10% fecal calf serum in Eagle minimal essential medium. After a monolayer was formed, the culture was maintained in 3% calf serum in Eagle minimal essential medium. WI-38 and BHK cell cultures (Flow Laboratories, Inc., Rockville, Md.) were maintained in 5% fetal calf serum in Eagle minimal essential medium.

Controls for the cytotoxicity tests were 10% aqueous extracts of stools from 14 patients receiving antibiotics without gastrointestinal complications, stools from 10 healthy individuals, and cecal contents from 10 healthy hamsters.

Inactivation procedures. The two specimens were tested under a variety of physical and chemical conditions for the effect on cytotoxicity. Heat inactivation was examined by using a water bath set at various temperatures in which the specimen was immersed for 10 min. The effect of pH and other conditions were determined at 37°C for 2 h; these included addition of 0.1 M boric acid (pH 3), glycine buffer (pH 10.4), ether, deoxyribonuclease (5 mg/ml), ribonuclease (1 mg/ml), and trypsin (0.25%). Dialysis was conducted by using 8-mm dialysis tubing (Union Carbide Corp., Chicago, Ill.) against 2 liters of 0.1 M phosphate buffer at pH 7.6 for 24 h in a refrigerator. Ammonia sulfate precipitation was tested by adding this reagent in 10% increments from 20 to 80%. At each step the precipitate was harvested and titrated for cytotoxicity.

Antitoxins. Inactivation by clostridia antitoxins was tested by incubating equal volumes of the antitoxin to each of the specimens at 37°C for 1 h. Commercially available gas gangrene antitoxin control no. 374-371 (American Cyanamid Co., Lederle Laboratories, Pearl River, N.Y.) was tested at a 1:10 dilution. This material contains 400 U against *C. perfringens* and *C. septicum*, 120 U against *C. histolyticum*, and 60 U against *C. novyi* and *C. sordellii*. Monovalent antitoxins, kindly supplied by V. R. Dowell, Jr., Center for Disease Control, Atlanta, Ga., included anti-*C. perfringens* type A, anti-*C. septicum*, anti-*C. histolyticum*, anti-*C. novyi* type A, and anti-*C. sordellii*, all standard antitoxins originally obtained from the Division of Biologics Standards, Rockville, Md. Monovalent antitoxins which neutralized the specimens were titrated by using serial twofold dilutions of the antitoxin against the specimen dilution to give 100 times the minimal detectable cytotoxicity. The reported titer was the highest dilution that gave no cytotoxicity. Equine serum was used as a control in these experiments, since all antitoxins were obtained from equine sources.

Cultures. Quantitative cultures were made from both specimens. Serial 100-fold dilutions were prepared with Virginia Polytechnic Institute dilution salt solution, and portions of 0.1 ml of the 10^{-4} , 10^{-6} , and 10^{-8} dilutions were plated with a glass spreader onto the following media: brucella base blood agar with 5 μ g of menadione and 10 μ g of hemin per ml (BMB), BMB containing 10 μ g of clindamycin hydrochloride per ml, Clostrisel (Scott Laboratories, Inc., Fiskeville, R.I.), and Clostrisel containing 10 μ g of clindamycin hydrochloride. Clostridial colonies were enumerated, subcultured, and identified according to criteria outlined in the *Anaerobe Laboratory Manual* of the Virginia Polytechnic Institute (4).

Clostridial isolates were grown under anaerobic conditions in EEG broth (2) for 5 days. The broth cultures were centrifuged at $2,000 \times g$ for 20 min, and the supernatant was inoculated into the cell culture. This cytotoxicity assay was also performed on the supernatants with and without gas gangrene polyvalent antitoxin or the *C. sordellii* standard antitoxin.

RESULTS

Cytotoxicity. Cytopathic changes varied with the three cell lines. WI-38 (human embryonic lung fibroblasts) and BHK cell cultures each showed cellular rounding with loss of cell processes. These changes were initially detected at 1 h and became prominent at 3 h. At higher dilutions of the inocula, the appearance of cell rounding was delayed and the degree of cell change was less complete. These changes mimic those produced by enterovirus. In human amnion cells, however, the affected cells showed actinomorphous changes, i.e., stringing of cell processes with radiation in all directions (Fig. 1). The actinomorphous feature remained for 2 to 3 days before cell rounding took place. These morphological features are distinct from those produced by enterovirus.

The human stools and the hamster cecal contents gave identical cytopathic effects with human amnion cells, and both were positive at 10^{-7} dilutions at 3 h. Incubation of toxin at 37°C showed that titers decreased by 1 log per day for 3 days. Titers with BHK and WI-38 cells were somewhat lower at 10^{-6} and 10^{-5} , respectively. All subsequent tests were performed with human amnion cells due to the high sensitivity and unique cytopathic effects.

Cytotoxicity tests of cecal contents from all 10 control hamsters and all 24 persons without intestinal disease showed no cytopathic effects.

Inactivation experiments. Reactions to physical agents are summarized in Table 1. It was noted that the fraction responsible for cytotoxicity in both specimens was acid labile, alkaline labile, ether resistant, and non-dialyzable. Exposure to 100°C for 10 min resulted in complete loss of activity; at 60 and 50°C the titer

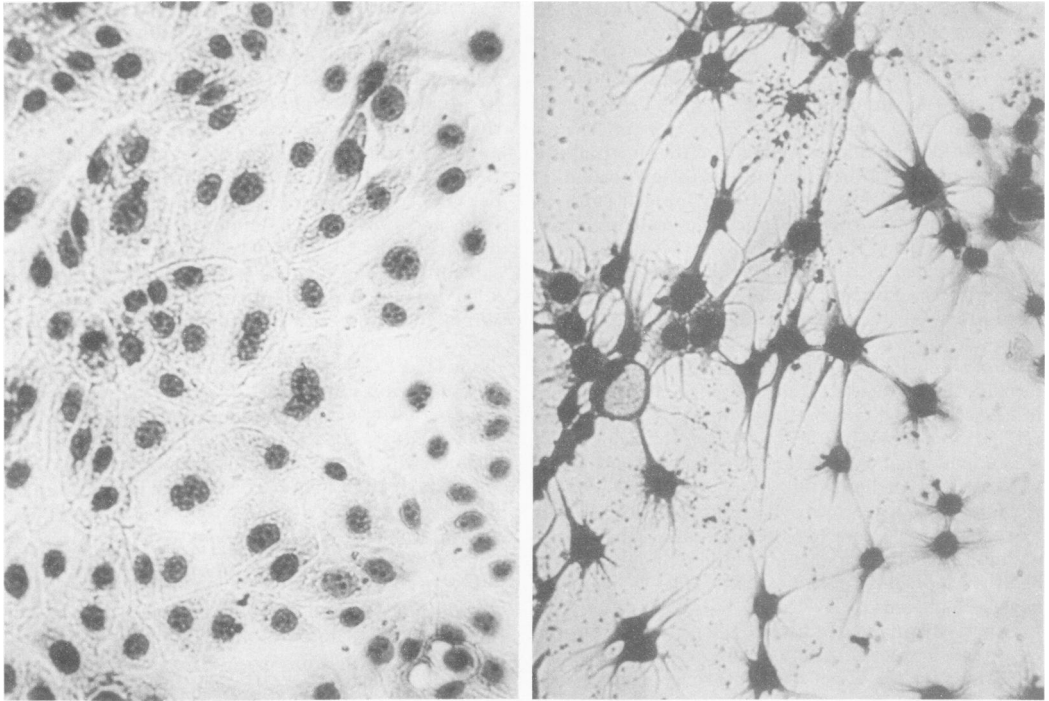


FIG. 1. Toxin-induced cytotoxicity of human amnion cells grown on cover glass. Giemsa stained. In contrast to the normal (left), toxin-treated cells (right) are stained dark with loss of detailed cellular structures and formation of stringing cytoplasmic processes radiating in all directions (actinomorph).

TABLE 1. Comparison of human stools and hamster cecal contents for cytotoxicity to human amnion cells

Characteristics	Human stools and hamster cecal contents ^a
Untreated	10^{-7}
pH 3 and 10.4	Inactivated ^b
Heat ($^{\circ}\text{C}$, 10 min)	
100	Inactivated
60	10^{-3}
50	10^{-5}
37	10^{-7}
Ether extraction	10^{-7}
Dialysis	Non-dialyzable
NH_4SO_4 precipitation	Peak activity at 60%
RNase, DNase ^c	10^{-7}
Trypsin	10^{-5}

^a Titer in cytotoxicity assay.

^b Inactivated indicates no detectable cytotoxicity.

^c RNase, Ribonuclease; DNase, deoxyribonuclease.

decreased from 10^7 to 10^3 and 10^5 , respectively. Ammonium sulfate precipitation produced a peak yield at 60% concentration. Tests of enzymatic digestion showed that both deoxyribonuclease and ribonuclease had no effect, whereas treatment with 0.25% trypsin for 2 h resulted in a 100-fold decrease in titer.

Neutralization by clostridial antitoxins.

Gas gangrene polyvalent antitoxin completely neutralized cytotoxicity in both specimens. Of the eight monovalent antitoxins, only the standard *C. sordellii* antitoxin was effective, and the titer with this antitoxin was 1:320 with both specimens. Equine serum, used as a control, had no effect on cytotoxicity.

Cultures. *C. difficile* was the predominant clostridium recovered in the stools from both the patient with PMC and from the cecal contents of the hamster with clindamycin-induced enterocolitis. These strains were recovered on media incorporating clindamycin as well as the other media employed. Quantitative cultures indicated concentrations of $10^{8.5}$ colony-forming units per ml in the hamster cecal contents and $10^{6.8}$ colony-forming units per g (wet wt) in the stools from the patient. Broth filtrates from these strains produced cytopathic changes in tissue cultures which were similar to those described with the stool and cecal specimens. These changes were noted in human amnion cells at 10^5 dilutions of the cell-free supernatants of the 48-h broth cultures. With both strains, cytotoxicity was neutralized by both polyvalent gas gangrene antitoxin and the *C. sordellii* standard antitoxin, but not by equine serum, as

described above. Four other species of clostridia were also recovered in the two specimens, but these were present in lower concentrations and failed to cause cytotoxicity.

DISCUSSION

A tissue culture assay was used to compare stools from a patient with autopsy-confirmed PMC and cecal contents from a hamster with clindamycin-induced enterocolitis. It was found that the specimens produced identical results in terms of cytopathic changes in all three types of cell cultures tested. However, we found that human amnion cells gave actinomorphous changes and higher titers compared to WI-38 cells. Consequently, we preferred human amnion cells, which were used for subsequent tissue culture testing. It was found that various physical and chemical treatments produced identical results with the specimens from humans and animals in this assay. With each, the material responsible for cytotoxicity was inactivated by acidification, alkalization, and heat; it was non-ether extractable and non-dialyzable; trypsin decreased activity 99%, and with NH_4SO_4 precipitation the peak activity was at 60%. These observations are most consistent with a protein toxin.

It was also noted that *C. difficile* emerged as a prominent component of the flora; this organism not only produced enterocolitis when injected intracecally into hamsters, but also produced typical cytopathic changes in the tissue culture assay. These results strongly suggest that this clostridial species was responsible for the cytopathic effects observed with the stools from the patient and the cecal contents from the hamster.

Of particular interest are the observations with the clostridial antitoxins. No *C. difficile* antitoxin was available for neutralization studies. However, both the gas gangrene polyvalent antitoxin and the *C. sordellii* standard antitoxin from the Bureau of Biologics neutralized the cytotoxicity. This suggests that either (i) *C. sordellii* and *C. difficile* produce a common toxin or share a toxic component or (ii) the *C. sordellii* antitoxin available for our studies was not, in fact, a monovalent antitoxin. Because the gas gangrene polyvalent antitoxin also neutralized

the *C. difficile* toxin, we were inclined to conclude that the two species produce the same toxin. However, since completion of these studies we have learned (L. V. Holdeman, personal communication) that two apparently different lots of *C. sordellii* antitoxin, received by the Virginia Polytechnic Institute Anaerobe Laboratory from L. S. McClung and labeled "serum 5389, 3-19-57" and "Lederle serum 5948B, 4-1-48," neutralize toxic supernatant fluid from two different *C. sordellii* cultures but not toxic supernatant fluid from two different *C. difficile* cultures. Since not all *C. sordellii* antitoxin does neutralize *C. difficile* toxin, perhaps the *C. sordellii* standard antitoxin and the *C. sordellii* component of the polyvalent gas gangrene antitoxin are not monovalent. Records indicate that both the polyvalent and the monovalent antitoxins were probably derived from the same culture (originating in 1930). The possibility that this is a mixture of *C. difficile* and *C. sordellii* is under investigation. We therefore feel that it is premature to make any firm statement about the antigenic relationship between the toxins of *C. sordellii* and *C. difficile*.

The results of this study indicate that clindamycin-associated enterocolitis in hamsters represents a useful model of human disease. Our findings also implicate a toxin produced by *C. difficile*, although it must be stated that our observations are limited to a single case and that other clostridial species might sometimes be involved.

LITERATURE CITED

1. Bartlett, J. G., and S. L. Gorbach. 1977. Pseudomembranous enterocolitis (antibiotic associated colitis). *Adv. Intern. Med.* 22:455-476.
2. Evans, D. K., D. G. Evans, and S. L. Gorbach. 1973. Production of vascular permeability factor by enterotoxic *Escherichia coli* isolated from man. *Infect. Immun.* 8:725-730.
3. Eyssen, H., P. DeSomer, and P. Van Dijck. 1957. Further studies on antibiotic toxicity in guinea pigs. *Antibiot. Chemoth. (Washington, D.C.)* 7:55-61.
4. Holdeman, L. V., and W. E. C. Moore (ed.). 1975. *Anaerobe laboratory manual*, 3rd ed. Virginia Polytechnic Institute and State University, Blacksburg.
5. Larson, H. E., J. V. Parry, A. B. Price, D. K. Davies, J. Dolby, and D. A. Tyrell. 1977. Underscribed toxin in pseudomembranous colitis. *Br. Med. J.* 1:1246-1248.
6. Small, J. G. 1968. Fatal enterocolitis in hamsters given lincomycin hydrochloride. *Lab. Anim. Care* 18:411-420.